Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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A 30-day follow-up study on the prevalence of SARS-COV-2 genetic markers in wastewater from the residence of COVID-19 patient and comparison with clinical positivity

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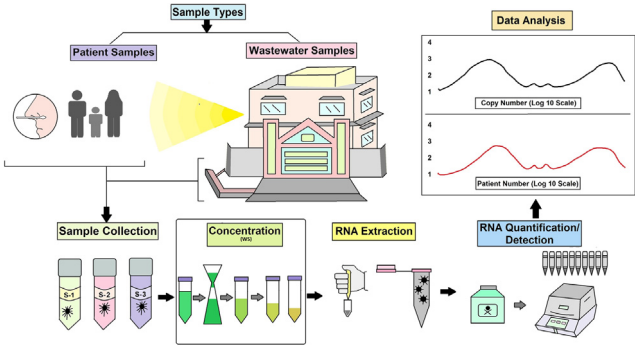
HIGHLIGHTS

- Wastewater SARS-CoV-2 RNA compared with COVID-19 clinical samples over a month
- An approach to identify high-prevalence locations of COVID-19 infection
- Positive correlation of patient number and SARS-CoV-2 genetic markers in wastewater
- Temperature and pH affected SARS-CoV-2 RNA load in wastewater.
- Delta variant (B.1.617.2) was detected from both clinical and wastewater samples.

GRAPHICAL ABSTRACT



Abbreviations: WBS, Wastewater Based Surveillance; WBE, Wastewater Based Epidemiology; RT-qPCR, Reverse Transcription-quantitative Polymerase Chain Reaction; WS, Wastewater Sample; CS, Clinical Sample; B.1.617.2, Delta Variants; COVID-19, Coronavirus Disease 2019; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2.

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ABSTRACT

Wastewater based epidemiology (WBE) is an important tool to fight against COVID-19 as it provides insights into the health status of the targeted population from a small single house to a large municipality in a cost-effective, rapid, and non-invasive way. The implementation of wastewater based surveillance (WBS) could reduce the burden on the public health system, management of pandemics, help to make informed decisions, and protect public health. In this study, a house with COVID-19 patients was targeted for monitoring the prevalence of SARS-CoV-2 genetic markers in wastewater samples (WS) with clinical specimens (CS) for a period of 30 days. RT-qPCR technique was employed to target non-structural (ORF1ab) and structural-nucleocapsid (N) protein genes of SARS-CoV-2, according to a validated experimental protocol. Physiological, environmental, and biological parameters were also measured following the American Public Health Association (APHA) standard protocols. SARS-CoV-2 viral shedding in wastewater peaked when the highest number of COVID-19 cases were clinically diagnosed. Throughout the study period, 7450 to 23,000 gene copies/1000 mL were detected, where we identified 47 % (57/120) positive samples from WS and 35 % (128/360) from CS. When the COVID-19 patient number was the lowest (2), the highest CT value (39.4; i.e., lowest copy number) was identified from WS. On the other hand, when the COVID-19 patients were the highest (6), the lowest CT value (25.2 i.e., highest copy numbers) was obtained from WS. An advance signal of increased SARS-CoV-2 viral load from the COVID-19 patient was found in WS earlier than in the CS. Using customized primer sets in a traditional PCR approach, we confirmed that all SARS-CoV-2 variants identified in both CS and WS were Delta variants (B.1.617.2). To our knowledge, this is the first follow-up study to determine a temporal relationship between COVID-19 patients and their discharge of SARS-CoV-2 RNA genetic markers in wastewater from a single house including all family members for clinical sampling from a developing country (Bangladesh), where a proper sewage system is lacking. The salient findings of the study indicate that monitoring the genetic markers of the SARS-CoV-2 virus in wastewater could identify COVID-19 cases, which reduces the burden on the public health system during COVID-19 pandemics.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has stemmed about 596.29 million confirmed cases and about 6.45 million deaths globally as of August 16, 2022 (WHO, COVID-19 Dashboard) (Islam et al., 2021; Sakib et al., 2021). Clinical diagnostic tests such as real-time polymerase chain reaction (q-PCR), quantitative reverse transcription PCR (RT-qPCR), rapid antigen and antibody test (RAT), and traditional serological tests are accepted as gold standard methods for detecting causative agents of many diseases including COVID-19 (Ahmed et al., 2021). Unfortunately, q-PCR and RT-qPCR tests for clinical diagnosis of COVID-19 that can detect viral genetic markers may take several days following the exposure of SARS-CoV-2 and are unable to detect pre-symptomatic and asymptomatic individuals (silent spreader of COVID-19) within the communities (Fig. 1) (Biggerstaff et al., 2014; Garg et al., 2020; C. Chakraborty et al., 2022). Following the infection, the SARS-CoV-2 virus and its various genetic components are shed through feces, urine, saliva, and other respiratory discharges from infected individuals, which are collectively designated as human waste (Wang et al., 2020). These human wastes and biological

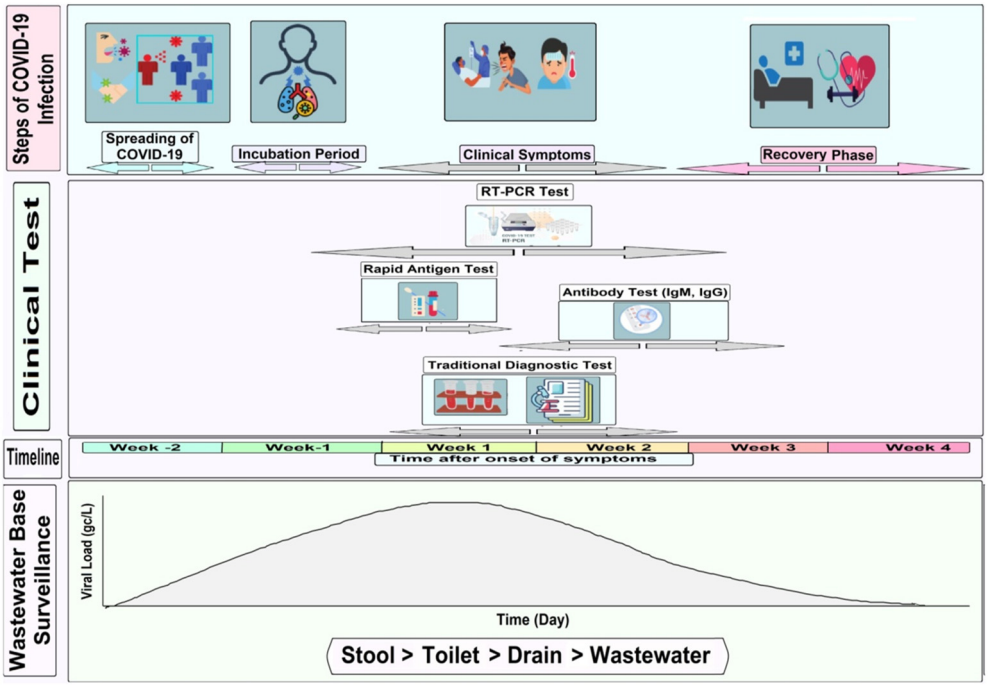

Fig. 1. Schematic diagram showing the timeline of COVID-19 diagnosis using clinical tests and wastewater-based epidemiology (WBE). The WBE can detect individuals before clinical testing as stool contains viral RNA. Clinical diagnostic tests mostly detect symptomatic patients seeking testing.

fluids containing the viral particles are discharged through wastewater outlets, during brushing, washing, sneezing, coughing, bathing or showering, washing clothes or hands, and wipes (Zheng et al., 2020).

Wastewater Based Epidemiology (WBE) has been reported as a convincing approach for tracking COVID-19 the pandemic through the identification of the hotspots and monitoring of the infection trends (Ahmed et al., 2021; Barceló, 2020; D'Aoust et al., 2021; Kumar et al., 2021a,b; Haramoto et al., 2020; Weidhaas et al., 2021; Wu et al., 2020; Jakariya et al., 2021). In addition, WBE can unravel the genetic markers of the viral RNA contributed by mild or asymptomatic patients and provides clinically unreported transmission episodes (Fig. 1). Furthermore, new mutations with genetic variants such as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) can also be tracked through WBE (Aleem et al., 2022). There are limited studies from developing countries that couple the concentration of SARS-CoV-2 viral biomarkers in wastewater with the identification of clinical cases in a specific residential area lacking wastewater treatment plants (Randazzo et al., 2020; Rakib, et al., 2021, 2022). However, the recovery of the SARS-CoV-2 viral RNA from wastewater is very challenging due to differential stability in wastewater streams, influenced by various environmental factors such as rainfall and temperature, as well as the presence of inhibitory substances (Ribonuclease Enzyme-RNase) (Farkas et al., 2018; Polo et al., 2020).

The present study aims to compare the number of clinically confirmed COVID-19 cases with the prevalence of SARS-CoV-2 RNA markers in wastewater samples based on a pilot investigation in a single household with clinically diagnosed patients and variants investigation. We tracked the main sources of SARS-CoV-2 RNA markers in wastewater from the patient's house and predicted COVID-19 cases with SARS-CoV-2 RNA in wastewater. To the best of our knowledge, this is the first follow-up study of COVID-19 wastewater coupled with the clinical samples from a single residence. This will be useful for several other developing countries like Bangladesh to predict COVID-19 cases prior to the clinical diagnosis.

2. Material and methods

2.1. Wastewater and clinical samples collection

Wastewater samples were collected daily for one month from the house of symptomatic COVID-19-positive patients located in the Noakhali district of southern Bangladesh (Fig. 2, Supplementary Table ST1), which had previously been confirmed by the Directorate General of Health Services (DGHS) report (Supplementary Fig. SF1). The sampling area is <1000 square feet with five toilets, two bathrooms, four sinks, four basins, and three kitchens with ~5 gal/day average wastewater flow. Among the twelve family personnel, three are babies (< 2 years), two are children (2-12 years), four are adults (12–70 years), and three are aged (>70 years) in the selected house.

Wastewater includes all the discharged water from toilets, showers, baths, basins and sinks, kitchens, as well as laundries. Four sampling sites covered all the drains of the selected house, where S1 was the main drain connected with feces and urines; S2 was linked with bathing outlets; S3 came from basin sand sinks; S4 was associated with household wastewater and kitchen outlets (Supplementary Table ST1). Composite samples were collected daily from sampling sites of the wastewater drain system from 22nd October to 20th November 2021. 100 mL of wastewater samples from 10:00 PM to 10:00 AM were collected and transferred to the NSTU laboratory in a sample transportation box. The samples were processed within 1 h of collection without any refrigeration. Relevant physico-chemical, environmental, and bacteriological data were recorded (Supplementary Fig. SF2). To prevent cross-contamination during transportation, autoclaved sample collection bottles were used. All the experiments and analyses were conducted at the COVID-19 Diagnostic Laboratory in the Department of Microbiology, NSTU, Bangladesh (an autonomous laboratory supported by Government of Bangladesh and quality controlled by the WHO Proficiency Testing Program). To assess the internal quality of

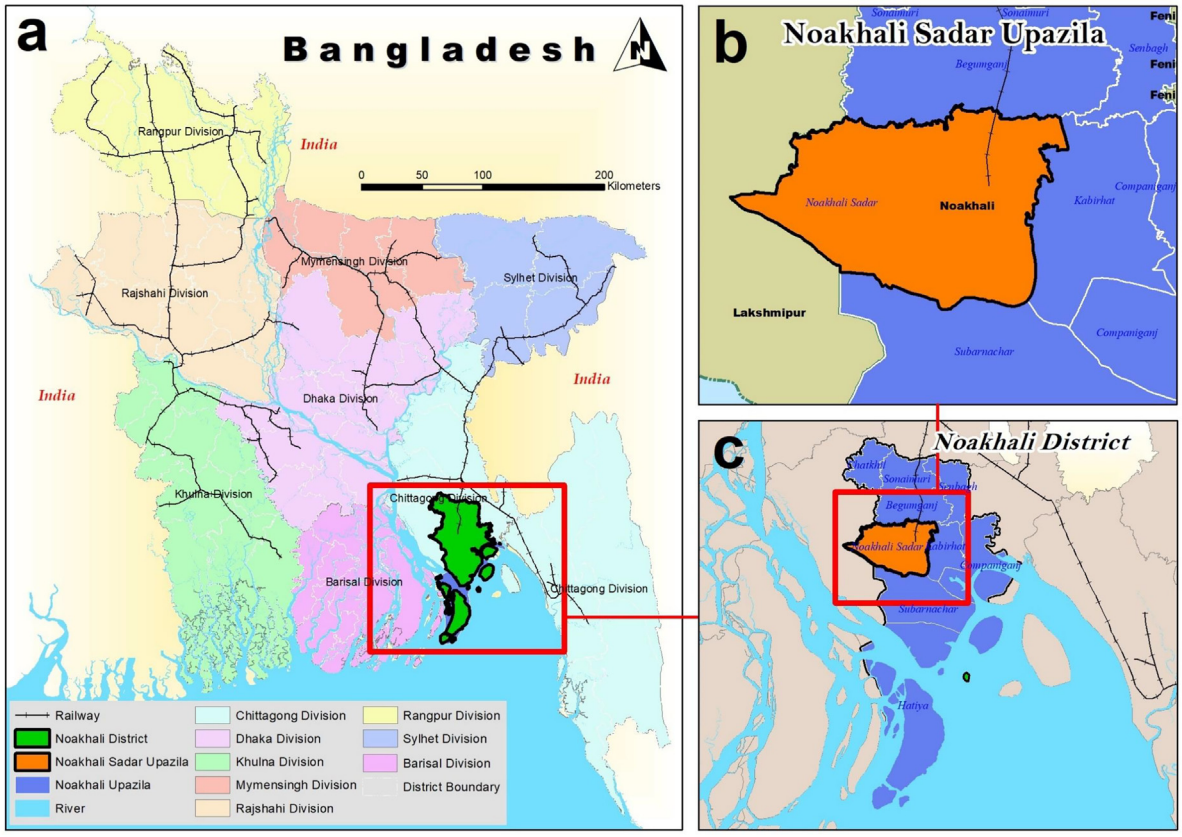

Fig. 2. a). Map of Bangladesh showing the study area in Noakhali district (marked red box); b) the selected house for sampling with SARS-CoV-2 positive patient; and c) The location of the study house in the Noakahli.

the laboratory, testing methods, and results, 5 % of samples were analyzed using RT-PCR in other laboratories [North-South University (NSU) Genomic Research Center and Jashore University of Science and Technology (JUST) Genome Center]. During the wastewater sample collection period, clinical samples from 12 persons in the selected house were collected daily by sterile nasal and oral swab with viral transport media (VTM) (Cat: NSTF90184; Invitrogen, UK) following the previously published protocol (Ahmed et al., 2021).

2.2. Ethical considerations

The study was reviewed and approved by the ethics committee of the Directorate General of Health Sciences (DGHS), Bangladesh, and by the National Research Ethics Committee (NREC) of the Bangladesh Medical Research Council (BMRC). Noakhali Science and Technology University (NSTU) Review Board for Human Subjects Protection looked over the work based on some criteria. NSTU COVID-19 Diagnostic Laboratory is a Bangladesh Government approved national COVID-19 testing center. Relevant demographic, clinical, and laboratory data were retrieved from the clinical records of the patient and signed written informed consents were obtained from participants and/or their legal guardians (Supplementary Tables ST2, ST3).

2.3. Environmental and physiochemical data

Environmental data were obtained from the database reported in the open information system on Worldmeter (Worldmeters.info, 2022), the official national data on WHO Coronavirus (COVID-19) Dashboard (WHO, 2022), and Bangladeshi public news reports. The analytical tests of the collected wastewater samples were performed as described in APHA (Ayaliew Werkneh, 2015) for different parameters such as temperature, pH, chemical oxygen demand (COD), dissolved oxygen (DO), conductivity, total suspended solids (TSS) and total dissolved solids (TDS). *E. coli* was counted using a standard microbiological procedure (Haque et al., 2022; Hossain et al., 2021a, 2021b).

2.4. Wastewater sample preparation and viral RNA extraction

All the wastewater samples (100 mL from each of the four sites) were filtered using a 0.22 μL syringe filter (Cat. No.231GE; Merck, USA Sartorius Products) and concentrated using Polyethylene Glycol (PEG-8000) (Fig. 3) following the published standard procedure (Chen et al., 2020; Ahmed et al., 2021; Kumar et al., 2021b). Total RNA was extracted from both concentrated wastewater samples and clinical samples using the QIAGEN Viral RNA extraction Mini Kit (CAT NO./ID 52940) according to the kit protocol. RNA quantity of all extracted wastewater samples checked by NanoDrop (Thermo Scientific TM Nanodrop 2000 and 2000c, BioRad). RT-qPCR (BioRad-CFX 96; CFX Maestro Software 2.2) was used to identify SARS-CoV-2 positive samples. To check internal laboratory quality, another RT-PCR equipment (Applied Biosystems™ Quant Studio 5 package-Thermo Fisher-Scientific; Software-Quant 5 Studio) was used in this study. For detecting SARS-CoV-2 positive patients, Directorate General of Health Services (DGHS), Bangladesh selected two genes (ORF1ab, N), with the human RNase P gene serving as the internal control (IC) for RT-PCR diagnostics. Hence, SARS-CoV-2 RNA from sewage samples and clinical samples were determined using the same genes. The human RNase P gene also served as an endogenous control indicating the validation of RNA extraction and the presence of inhibitors in the wastewater samples, which is a standard gene fragment with all of the RT-PCR runs to detect the human RNase P gene.

Predominantly, SARS-CoV-2 genetic components were detected using a commercial RT-PCR kit (Sansure Biotech Inc., China) (Cat: 034BF234), and the results were interpreted according to the kit protocol (Doc. #: 2019-nCoV IFU). In summary, we used a 45-cycle RT-PCR technique to detect fluorescent FAM dye for ORF1ab, ROX dye for the N gene, and CY5 for human RNase P (Supplementary Table ST4). RT-PCR reactions were run

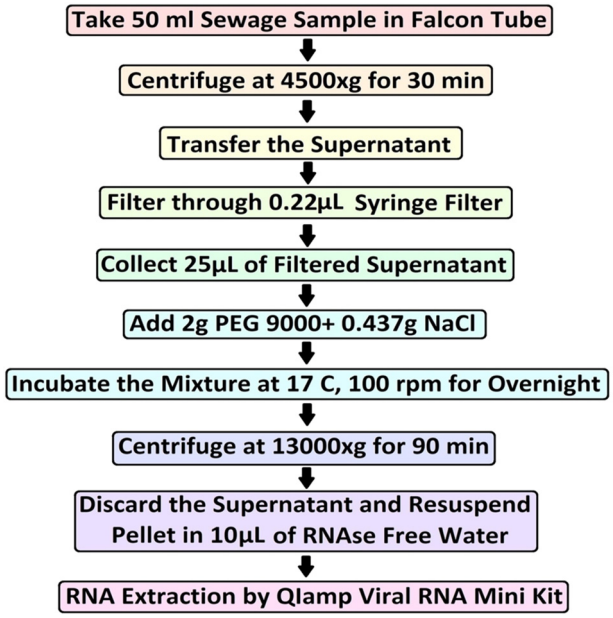

Fig. 3. Optimized method for the concentration of the SARS-CoV-2 RNA using PEG following the methods described earlier (Chen et al., 2020; Ahmed et al., 2021; Kumar et al., 2021b).

on a CFX96 Touch Real-Time PCR Detection System at 50 °C for 30 min, then 47 cycles of 95 °C for 10 s and 60 °C for 30 s. The sample was selected as positive for both WS and CS if the cycle threshold was below or equal to 40 cycles. To validate the Sansure RT-PCR kit, we carried out SARS-CoV-2 gene quantification with another commercial kit (BGI; 2019-nCoV RT-PCR kit) to test 5 % positive and negative samples at random and found comparable results. SARS-CoV-2 genes were quantified using 10-fold dilutions (range of 1.0E + 02 to 1.0E + 05 copies per assay) with the 2019-nCoV N plasmid DNA as known positive control from IDT (Leuven, Belgium).

2.5. Estimation of SARS-CoV-2 cases from wastewater samples

To estimate the prevalence of COVID-19 cases by analyzing wastewater samples, we followed a formula based on the average number of RNA copies in four sampling sites (Ahmed et al., 2020). Eq. (1) was used to estimate COVID-19 patients where feces excreted/person/day = 128 g was assigned (Rose et al., 2015). The Monte Carlo method was employed to determine the number of SARS-CoV-2 RNA copies shed in stool by infected people using Oracle Crystal Ball (Release 11.1.2.4.600, Redwood City, CA). The daily wastewater was assumed to be an average of 22.5 L/day (Okoffo et al., 2019).

$$\text{Infection number} = \frac{\left(\frac{RNA copies}{liter\ wastewater}\right) \times \left(\frac{liter\ wastewater}{day}\right)}{\left(\frac{g\ feces}{\frac{person}{day}}\right) \times \left(\frac{RNA\ copies}{g\ feces}\right)} \quad (1)$$

2.6. Identification of SARS-CoV-2 variants

The cDNA was synthesized from viral RNA using the SuperScriptTM III First-Strand Synthesis System (InvitrogenTM, Thermo Fisher Scientific, USA) at the COVID-19 Diagnostic Laboratory, NSTU and Genome Center, JUST. The cDNA concentrations were measured using the dsDNA HS Assay Kit combined with Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). In this study, New England Biolab 2× master mix and designed primer sets were used (Table 1), and 50 μL of PCR reaction volume was

Table 1
Primer sets used for SARS-CoV-2 variants in this study.

Name	Sequence	Product size (bp)	Tm (°C)	Reference
WV	F:CTCCAGGGCAAACTGGAAAG R:CAGTTGCTGGTGCATGTAGAA	338	54 °C	This study
IV-1	F: GCACACCTTGTAATGGTGTTC R:GGGACTTCTGTGCAGTTAACAC	390	51 °C	
IV-2	F:GGTTGGTGGTAATTATAATTACCG R:CCTTCAACACCATTACAAGGTT	78	51 °C	
SAV-1	F: CTCCAGGGCAAACTGGAAAT R:GGACTTCTGTGCAGTTAACAC	629	53 °C	
SAV-2	F: GCACACCTTGTAATGGTGTTA R: GGTTGGTAACCAACACCATA	90	48 °C	
UKV-1	F: GCACACCTTGTAATGGTGTTA R: GGACTTCTGTGCAGTTAACAC	392	48 °C	
UKV-2	F: CATATGGTTTCCAACCCACTT R:GGACTTCTGTGCAGTTAACAC	341	46 °C	

Note: WV = Wuhan/Conventional Variants; IV = Indian Variant; SA = South African Variant; UKV = United Kingdom Variant.

performed in a T100 Thermal cycler (Bio-Rad, United States) using a validated annealing temperature, then confirmed by 2 % gel electrophoresis using Bio-Rad Gel Documentation system (Supplementary Table ST4). Primers for SARS-CoV-2 variants were designed using previously submitted data sets from GISAID (https://www.gisaid.org/). Samples sequenced by whole-genome sequence (GISAID Accession ID-EPI_ISL_1626483 to EPI_ISL_16264527, EPI_ISL_2036272, EPI_ISL_2350142, EPI_ISL_234980 submitted by COVID-19 Diagnostic Lab, NSTU with NSU Genome Research Center and JUST Genome Center were used for primer validation (Hossain et al., 2021a, 2021b).

2.7. Results validation and quality control

To avoid cross-contamination during sample collection and transportation, one sample collection bottle was filled with normal saline and checked during the RT-PCR run; positive, negative, and no-template controls were also taken as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Tiwari et al., 2021; Huggett et al., 2013). An extraction control was applied with a positive and negative COVID-19 patient sample. To check and verify RT-PCR inhibition, a known sample (Positive standard sample confirmed by RT-PCR from three different labs) was used in each run. If the results were similar (acceptable 5 % deviation) with the previously determined positive and negative samples by RT-PCR, all samples were used for further analysis. As described in the standard protocol, for viral shedding, 100 mL wastewater samples were concentrated using the precipitation method. Then, a positive clinical sample for SARS-CoV-2, with 7.3×10^6 gene copies/L (GC/L) was employed. The recovery efficiency of SARS-CoV-2 was assessed in this study using bovine coronavirus (BCoV) based on its gene copies quantified by RT-qPCR (Joshi et al., 2022) (Table 2). To verify primer-primer dimer and false-positive results, a melt curve was used before the analysis.

Table 2
SARS-CoV-2 recovery from sewage samples concentrated by PEG precipitation.

SARS-CoV-2 seeded (GC/L)	SARS-CoV-2 recovered genes		Mean concentration (GC/L) ± SD[a]	Mean recovery %
	ORF1ab	N		
7.3×10^3	(2/3)	(2/3)	3.6×10^3-6.1×10^3	66.12
7.3×10^2	(3/3)	(2/3)	4.4×10^2-5.9×10^2	70.32
7.3×10^1	(1/3)	(3/3)	3.3×10^3–5.2×10^3	58.43

Note:
[a] SD = Standard Deviation.

2.8. Data analysis and cost calculation

Quantitative variables were summarized using mean and standard deviation (SD). The association between quantitative variables was calculated using the Pearson correlation coefficient and linear regression. Statistically, a significant difference was judged as $^*p < 0.05$ and $^{**}p < 0.01$. The statistical analysis was carried out using the R programming tool and SPSSv.25. Wastewater and clinical sampling, experiments, and analysis costs were calculated using standard prices of reagents.

3. Results

3.1. Detection of SARS-CoV-2 genetic markers in the wastewater and clinical samples

Overall, 47 % (57/120 of the total samples) of wastewater samples and 35 % (128/360 of the total samples) of clinical samples were positive for the two assayed SARS-CoV-2 genetic RNA markers (ORF1ab or N). The results indicate that the SARS-CoV-2 positivity rate is higher in wastewater rather than in clinical samples ($p = 0.020$). However, both ORF1ab and N genes of SARS-CoV-2 were detected in 8 samples (7 %) of wastewater and 65 (18 %) clinical samples. On the contrary, the observation showed that SARS-CoV-2 RNA genetic markers in the clinical samples were consistently stable ($p = 0.002$) than in wastewater samples, which could be due to the availability of RNase enzymes and other cofactors in the environment (Jakariya et al., 2022). In the wastewater samples, only nucleocapsid (N-gene) region was found in 31 (65 %), the Nonstructural region (ORF1ab gene) in 21 (44 %), and the internal control human genes (RNase P gene) in 8 samples (17 %) (Fig. 4). In addition that, 100 % IC-internal control gene (RNase P gene), 85 % N genes, and 30 % ORF1ab genes were determined from clinical samples of the positive patient's house respectively (Fig. 4). The RNase P gene detected the presence of human genes in collected wastewater samples, indicating that the RNase P gene was common in all the clinical samples, where 17 % were identified in wastewater samples.

From the clinical samples, the highest CT values detected for the ORF1ab gene (39.37), N gene (39.00), and RNase P gene (39.48), while the lowest observed CT values were 26.14, 26.28, and 25.45. When analyzing the day-wise prediction, the lowest SARS-CoV-2 RNA was observed during the last week of sample collection as patient numbers declined. In wastewater samples, the highest CT values for the ORF1ab, N and RNase P genes were 39.4, 39.65 and 39.7 respectively while the lowest corresponding CT values were 32.11, 31 and 25.2 respectively, which was obtained during the third week of sampling. Prior to the data analysis, positive control, negative control, extraction control, and no template control (NTC) are checked (see Supplementary Figures SF3, SF4). From four sampling sites, maximum positive samples were found in site 1 which was obtained from urine and feces connected with toilets (Jones et al., 2020) (Supplementary Table ST5). We identified the potential sources of SARS-CoV-2 RNA captured from four sampling points of the selected house (Supplementary Table ST5). Compared to the other three points, we found the highest percentage of SARS-CoV-2 positive samples in S1 (50%) and the lowest in S3 (30%) (Li et al., 2022). Our study findings matched with previous similar studies carried out for the detection of SARS-CoV-2 RNA in the patient's bodily fluids such as blood, feces, urine, saliva, and sputum (Peng et al., 2020).

3.2. Detection of increased SARS-CoV-2 RNA biomarkers in wastewater and early prediction of COVID-19 patients

In this study, we observed a high positivity rate in wastewater samples, with 75 % positive samples being reported between November 3rd and November 9th, 2021, followed by a decline in SARS-CoV-2 positive samples. The SARS-CoV-2 positive rate in clinical samples ranged from 16 % (2/12) in the first week of monitoring to a maximum of 50 % (6/12) in

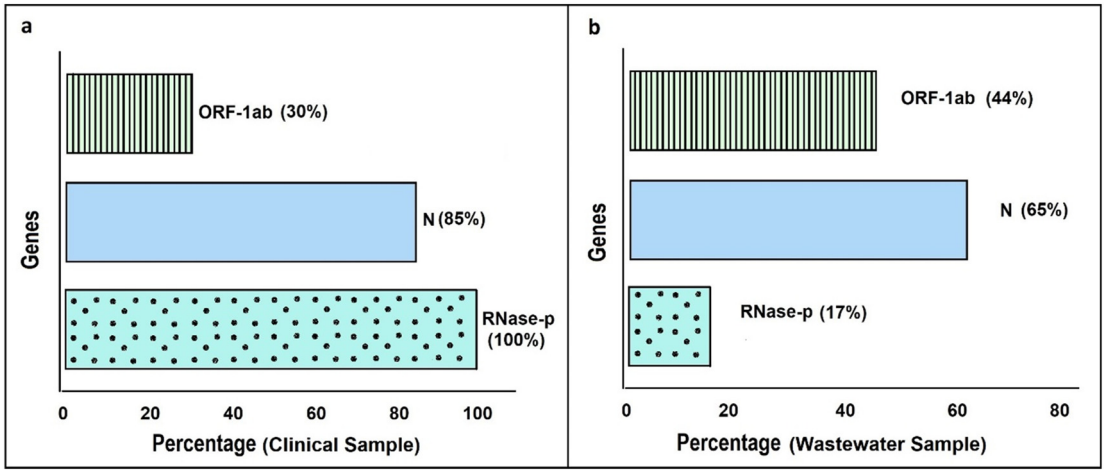

Fig. 4. Percentage of SARS-CoV-2 and internal control genes (ORF1ab, N, and RNase P) in a) clinical samples and b) wastewater samples.

the 3rd and early half of the 4th week, before reverting to 25 % at the end of the study (Table 3). Notably, the rising trend of the positive rate in wastewater samples was similar to the positive rate observed in clinical samples. This result suggests that wastewater monitoring can be used for the early detection of COVID-19 infection and hotspots. Wastewater monitoring can thus indicate a trend of the prevalence of COVID-19 in a community.

The results indicated that the positivity rate of both assays of SARS-CoV-2 RNA (ORF1ab, N) was higher in WS than in clinical samples (Wu et al., 2020).

3.3. Environmental factors

SARS-CoV-2 gene copy numbers correlated with temperature and pH in wastewater is presented in the Fig. 5. An increase in temperature was linked to a decrease in cycle threshold values ($p = 0.001$), especially for the ORF1ab and N genes. Most of the SARS-CoV-2 assays were detected at a temperature ranging from 27 to 35 °C (Table 4). Similarly, SARS-CoV-2 RNA was more frequently detected in the neutral to alkaline pH (6.23–11.89) ranges. The temperature of wastewater was strongly correlated with CT values (Fig. 5). This study was conducted during the summer season when the lowest temperature was 27.3 °C. Hence, winter season or cold days were not considered with COVID-19 cases. Notably, another study evaluated the effect of temperature on SARS-CoV-2 RNA in wastewater, where most of the findings were similar to our study results (Weidhaas et al., 2021).

3.4. SARS-CoV-2 CT value versus COVID-19 confirmed cases

The number of COVID-19 positive cases was linked with SARS-CoV-2 gene copy number monitored in wastewater (Fig. 6). When the number of SARS-CoV-2 positive patients was lowest (two positive subjects), the highest CT value of 39.4 was recorded in wastewater samples with an average copy number of 7450/1000 mL of wastewater. In contrast, the lowest CT value of 25.2 was recorded when the number of patients was the highest (six positive subjects) with copy number 23000/1000 mL (Fig. 6). Further, we found four positive patients from wastewater on the first day, with the number increasing until the 15th day, then decreasing. We also noticed that the number of calculated positive cases from wastewater increased higher than the number of positive cases from the clinical sample and decreased more slowly. We can deduce that wastewater exhibited the patient's positivity prior to the clinical tests, based on our COVID-19 case estimation result.

3.5. Detection of the genetic markers of SARS-CoV-2 variants

The Delta variant was found positive in all assayed CS collected from the patients of the target household. In addition, we looked into other variants of concern (VoC), but six wastewater samples (WS-12, WS-20, WS-23, WS-26, WS-43, WS-64) tested positive for the Delta variant (Supplementary Fig. SF3) ensuring positive control (the whole-genome sequenced samples). This study indicated that surveillance of wastewater is an approach that allows monitoring the diversity of SARS-CoV-2 variants circulating in the community (Nag et al., 2022). We designed primer sets for variants of interest (VoI) of COVID-19 targeting mutation points which were confirmed by conventional PCR test (Supplementary Fig. SF4). Our findings indicated that L452R and T478K mutations are available in Delta (B.1.617.2) variants. It was also observed that the L452R mutation of COVID-19 enhanced the infectivity and evaded the cellular immunity of patients (Suchard et al., 2018; Sakib et al., 2021). Additionally, L452R helped in decreasing the binding of specific monoclonal antibodies (mAbs) with neutralization. Another common mutation of the Delta variant, T478K, was possibly associated with increasing ACE2 binding sites, which helps to increase transmissibility (Zhang et al., 2022). This study also demonstrated that the Delta variant of SARS-CoV-2 was found as the dominant variant in the clinical samples and found to be more transmissible (60 %) during the study period (Joshi et al., 2022).

Table 3
Comparision of the percentage of positive SARS-CoV-2 genes in wastewater samples (WS) and clinical samples (CS).

Sampling Date	October 2021										November 2021																						
	22	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			
Positive Rate in CS	16%						25%				41%				50%									33%				25%					
Positive Rate in WS	25%										50%													75%							50%		25%

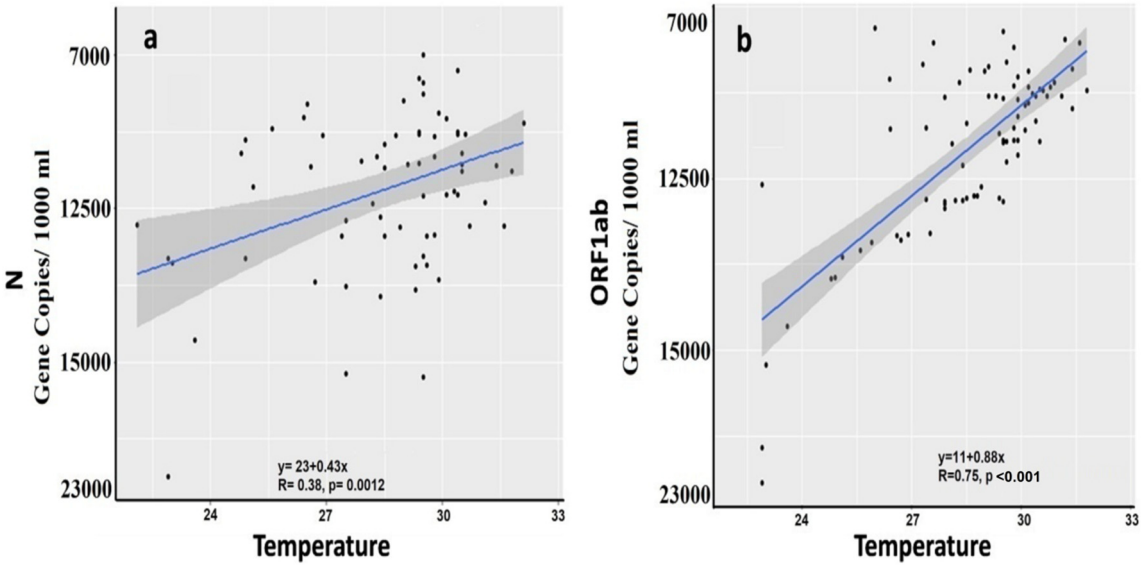

Fig. 5. Correlation of physicochemical parameters with the abundances of the genetic markers of SARS-CoV-2 in wastewater. a) correlation of temperature (Y-axis) with N gene (X-axis); b) correlation of temperature (Y-axis) with ORF1ab gene (X-axis).

3.6. Cost calculation of WBS

WBE surveillance is more affordable (60 $/day) than clinical sample surveillance (419 $/day) for predicting the COVID-19 pandemic (Supplementary Table ST6) (Esbin et al., 2020; Won et al., 2020). Although the sample preparation and RNA concentration need extended time, the overall procedure was more accessible and completed within 5–6 h. The pilot cost calculation report of this study indicated that WBE could be employed for monitoring of COVID-19 pandemic in low-income developing countries (Jakariya et al., 2021; Zhang et al., 2022).

4. Discussion

According to our results, CT value and SARS-CoV-2 gene copy numbers in WS can be used to evaluate COVID-19 trends, which will eventually help to predict the number of COVID-19 patients (Daughton, 2020; Tiwari et al., 2022a). Based on the RT-qPCR test results, the CT values (25–39) in wastewater, it can be concluded that the range varied due to the changes in the infection rate which supports previous data (Mlejnkova et al., 2020). One previous study in Massachusetts, USA between March and May 2020 reported that the amount of SARS-CoV-2 RNA biomarkers in wastewater followed the same trend as COVID-19 patients numbers (Wang et al., 2020). Another study in Utah, USA used 9-week WS sampling and reported a link between a community outbreak and an increase in SARS-CoV-2 RNA (Weidhaas et al., 2021). As in our study there was no wastewater treatment system in the selected house and no interlinkage with other drain systems, all the SARS-CoV-2 RNA in the wastewater samples represented the shedding from 12 persons in the house. We also observed an increasing trend of SARS-CoV-2 RNA in WS with the increased number of COVID-19 patients in the household as well as time-dependent decrease in the copies of SARS-CoV-2 RNA genetic markers with the recovery of patients (Supplementary Table ST6).

This study also reveals that SARS-CoV-2 RNA appears to be damaged or destroyed more frequently in WS than in CS (Ahmed et al., 2021; Tiwari et al., 2021). In CS, Nucleocapsid protein genes (N gene) were less damaged than nonstructural protein genes (ORF1ab), and the percentage of the internal control gene, RNase P, was higher. The causes of higher persistency of N gene than ORF1ab were unclear and further research may needed for evaluating this. The SARS-CoV-2 detection percentage was higher in WS than in CS (47 % > 35 %), and an increasing trend was noticed first in WS than in CS. These results support that WBE can work as a reliable early warning tool for COVID-19. Some earlier WBE studies argued for the early detection of SARS-CoV-2 RNA from WS than COVID-19 cases in communties (Kumar et al., 2021a). Medema et al. (2020) reported the SARS-CoV-2 genetic marker in WS in February, which was before the official confirmation of the first clinical case in the Netherlands. Similarly, La Rosa et al. (2020) detected SARS-CoV-2 genes in WS before the first official report for the clinical cases in two cities of Italy. Environmental parameters are also linked to SARS-CoV-2 RNA markers, as evidenced by a decrease in the viral gene

Table 4
Physiochemical and biological parameters of wastewater.

Parameter		Maximum	Minimum	Mean ± SD	*P*-value
Physicochemical	pH	11.89	6.23	7.64 ± 1.37	0.04*
	Temperature(°C)	35.6	27.01	30.40 ± 61.66	0.03*
	TDS (ppt)	7.62	2.16	5.149 ± 1.74	0.67
	TSS (mg/L)	14.00	6.65	7.45 ± 8.00	0.28
	EC (mS/m)	10.84	1.40	6.27 ± 1.40	0.33
	Salinity (ppt)	5.17	1.20	3.62 ± 1.20	0.54
	COD (mg/L)	98.00	15.00	55.33 ± 15.00	0.12
	DO (mg/L)	5.23	1.40	3.07 ± 1.40	0.65
Biological	*E. coli*/100 mL	651.00	120.00	292.16 ± 120.00	0.12
Environmental (air)	Temperature(°C)	38.00	18.00	25.00 ± 2.12	0.21
	Rainfall (mm/h)	6.10	0.10	2.40 ± 0.76	0.53
	Humidity ($g.kg^{-1}$)	96.00	49.00	63.00 ± 1.50	0.65

Note: SD = Standard deviation; TDS = Total dissolved solid; TSS = Total suspended solid; EC = Electric Conductivity; COD = Chemical oxygen demand; DO = Dissolved oxygen.

* Significant at 5 % level.

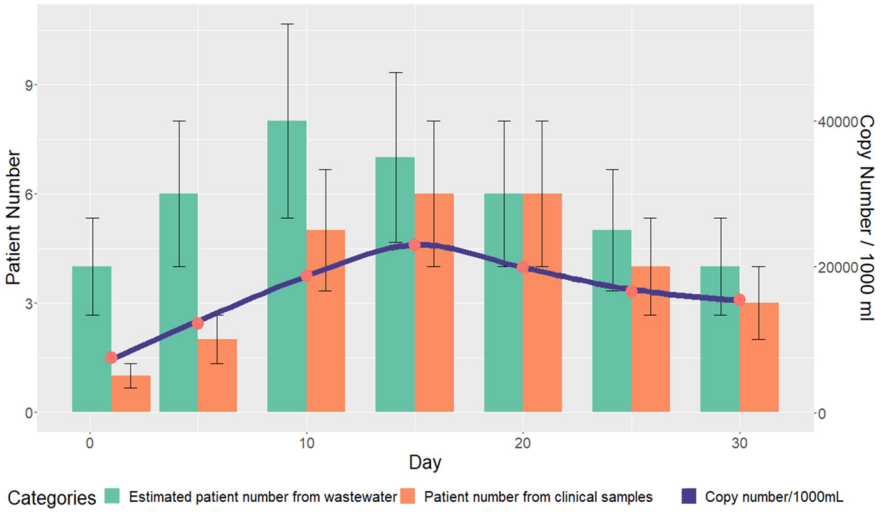

Fig. 6. Gene copy number of SARS-CoV-2 in wastewater correlated with COVID-19 patient number. The trend line shows the average gene copy number/1000 mL in wastewater, and bar charts show the COVID-19 patients detected concurrently with wastewater sampling.

copy numbers with increasing wastewater temperature (Bardi and Oliaee, 2021). In a previous study, the concentrations of the SARS-CoV-2 biomarkers in wastewater samples in Canada (Ottawa) surged by >400 % within 48 h following >300 % rise in the number of clinically diagnosed cases (D'Aoust et al., 2021). In another study in Utah, a strong link was observed between community outbreaks and increase in SARS-CoV-2 RNA in wastewater (Weidhaas et al., 2021). From the four sampling sites in our study, we have observed maximum gene copies (17,000/1000 mL) carried in the two large drains of the house (Ahmed et al., 2021).

COVID-19 can be diagnosed using a variety of clinical laboratory tests (Alkhateeb et al., 2021), however implementing these could be difficult for various factors, such as lack of consumables, and shortage of reagents with high cost, ambiguity, and a lack of monitoring experts. Another major problem is that viral particles are exhibited later in feces than in a clinical sample. These obstacles prompted the development of different epidemiological methods (Foladori et al., 2020). WBE can be used to detect asymptomatic and pre-symptomatic persons because they both excrete the virus with feces. The current study result also supports WBE as a complementary tool because we were also able to detect SARS-CoV-2 variants from WS, where all detected variants were delta variants. We observed that WBE surveillance is less time consuming and needs lower cost than CS, as this study required 3× CS (Number of Clinical Sample = 360, Wastewater Sample = 120) than WS and 7 times higher cost for CS than WS (Esbin et al., 2020).

We found SARS-CoV-2 RNA in all four sampling sites, with urine and feces accounting for 35 % of positive samples, and this sampling site connected directly with toilets indicating that toilet wastewater can be used for sampling. Other sampling sites linked with bathing outlets, basins, sinks, and kitchen outlets can also be used as secondary sources. The lowest percentages of positive samples were found in basins and sink wastewater when soap, hand wash, and other disinfectants were used. Positive samples were found only from toilet wastewater for the first three days. SARS-CoV-2 genetic material and its propensity for dissemination are not uniformly distributed across the country. To determine the pandemic trend, wastewater samples must be analyzed at regular intervals over a longer period. The seasonal variation must also be considered, as it significantly impacts the propagation of harmful viruses and bacteria. To our knowledge, this follow-up study from non-point waste water sources without wastewater treatment plants showed significant results that will help developing countries like Bangladesh to identify COVID-19 cases earlier than the clinical test (Islam et al., 2022b).

This current study suggests that wastewater surveillance could be useful for monitoring the COVID-19 pandemic and the same strategy could be followed for other infectious viruses (C. Chakraborty et al., 2022; Chakraborty et al., 2022a–c; Chandran et al., 2022; Dhama et al., 2022). However, more research is required to determine the link between COVID-19 symptom severity and SARS-CoV-2 RNA shedding in fecal samples and eventually wastewater discharges (Islam et al., 2022a,b; Tiwari et al., 2022a). The concentration of viral RNA in wastewater can be affected by environmental variables such as temperature and pH, as these factors can affect the decay rate of SARS-CoV-2 RNA (Tiwari et al., 2022b). The study also illustrates that the WBE can cover all four aspects of SARS-CoV-2 surveillance, including early warning, monitoring of propensity trends, genetic diversity, and prevalence of the SARS-CoV-2 variants in rural areas lacking proper sewage or drainage system.

5. Conclusion

This study compared the prevalence of SARS-CoV-2 RNA (ORF1ab and N genes) in wastewater with the clinical samples from COVID-19 patients for one month in a house where COVID-19 clinial cases was diagnosed. The concentration of SARS-CoV-2 RNA markers in wastewater from the various outlets were related to the number of COVID-19 affected individuals in the household. However, the temporal variations in SARS-CoV-2 RNA concentrations need to be further investigated from multiple perspectives. WBE can be used as a useful tool to estimate COVID-19 patients at the community level, especially in developing countries with limited clinical diagnostic facilities. WBE needs to be integrated with other public health services, namely campaign-based and randomized testing of individuals (presence of COVID-19), clinical testing, web contact tracing, and self-diagnostic reporting systems. Therefore, it is necessary to develop a validated method for predicting COVID-19 patient numbers from SARS-CoV-2 viral shredding in wastewater compared to clinically confirmed positive cases. The COVID-19 pandemic in Bangladesh is now in its third stage but based on the experiences of the other countries, further waves of infection may be foreseen; therefore WBE-based surveillance system involving community-level health management would help to control the future outbreaks of the pandemic at scale.

CRediT authorship contribution statement

Md. Aminul Islam: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Roles/Writing - original draft, Writing - review & editing.

Md. Arifur Rahman: Visualization, Writing - review & editing.

Md. Jakariya: Conceptualization, Funding, Project administration, Supervision.

Newaz Mohammed Bahadur: Writing - review & editing.
Foysal Hossen: Data curation, Investigation, Visualization.
Sanjoy Kumar Mukharjee: Writing - review & editing.
Mohammad Salim Hossain: Writing - review & editing.
Atkeeya Tasneem: Data curation, Investigation, Visualization.
Md Atiqul Haque: Data curation, Investigation, Visualization.
Francesco Sera: Writing - review & editing.
Iqbal Kabir Jahid: Writing - review & editing.
Tanvir Ahmed: Funding, Writing - review & editing.
Mohammad Nayeem Hasan: Writing - review & editing.
Md. Tahmidul Islam: Project administration, Funding.
Md. Amzad Hossain: Writing - review & editing.
Md. Ruhul Amin: Writing - review & editing.
Ananda Tiwari: Writing - review & editing.
Md. Didar-ul-Alam: Writing - review & editing.
Kuldeep Dhama: Writing - review & editing.
Prosun Bhattacharya: Conceptualization, Funding, Project administration, Roles/Writing - original draft, Writing - review & editing, Supervision Project administration.
Firoz Ahmed: Conceptualization, Project administration, Writing - review & editing, Supervision Project administration.

All authors critically scrutinised and approved the final version of the manuscript. The corresponding authors are responsible for confirming that the descriptions are accurate and agreed by all authors.

Ethical statement

This study was approved and reviewed by Ethical Reviewing Board at Noakhali Science and Technology University. Directorate General of Health Sciences (DGHS), Bangladesh, approved clinical sample collection based on some conditions (BMRCAIREC/2021/ I 708).

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Data availability

The authors do not have permission to share data.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159350.

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